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ANTIBODY TO IL-12 RECEPTOR

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ANTIBODY TO IL-12 RECEPTOR

BACKGROUND OF THE INVENTION

IL-12, formerly known as cytotoxic lymphocyte maturation factor, is a cytokine that stimulates proliferation of PHA-activated human peripheral blood lymphoblasts and synergizes with low concentrations of IL-2 in the induction of lymphokine-activated killer cells. IL-12 is a 75-kDa heterodimer composed of disulfide-bonded 40-kDa and 35-kDa subunits. Monoclonal antibodies have been prepared against a partially purified preparation of natural IL-12. These antibodies have been characterized by (1) immunoprecipitation of ^{125}I-labeled IL-12, (2) immunodepletion of IL-12 bioactivity, (3) Western blotting of IL-12, (4) inhibition of ^{125}IL-12 binding to its cellular receptor, and (5) neutralization of IL-12 bioactivity. It was determined that antibodies specific for the 40-kDa subunit of IL-12 block receptor binding of ^{125}IL-12 and neutralize IL-12 bioactivity. See in this regard Chizzonite *et al.*, J. Immunol. 147:1548 (1991).

The initial characterization of the IL-12 receptor has been reported for mitogen- and IL-2-activated human peripheral blood mononuclear cells (PBMC) and tonsilar lymphocytes. Radiolabeled IL-12 binding assays demonstrated that at the time of peak expression, mitogen- or IL-2-activated cells expressed 1000 to 9000 IL-12 binding sites/cell with a K_D of approximately 100 to 600 pM. The variations in K_D and sites per cell were dependent on the individual preparations of lymphoblasts. The binding of ^{125}I-labeled IL-12 to PHA-activated PBMC was saturable and specific, since the binding of radiolabled ligand was only inhibited by IL-12 and not by other cytokines. Kinetic studies revealed that maximum

expression of IL-12R occurred earlier on PHA-activated PBMC as compared with PBMC activated by IL-2, and that expression of IL-12R on these cells correlated with their ability to proliferate in response to IL-2. See Chizzonite *et al.*, J. Immun l. 148:3117 (1992) and Desai *et al.*, J. Immun l. 148:3125 (1992). Summing the results obtained in these two papers, activation of T cells or NK cells results in up-regulation of IL-12R expression; on the other hand, B cell activation, at least under some circumstances, appears not to be associated with enhanced expression of IL-12R.

SUMMARY OF THE INVENTION

The present invention relates to novel antibodies against the IL-12R. Representative anti-IL-12R antisera provided in accordance with the present invention block IL-12 binding to cells expressing IL-12R and can also neutralize IL-12 activity. In further embodiments of the present invention, monoclonal antibodies which are selective to IL-12R are prepared in accordance with generally known techniques, such as the method of Kohler and Milstein. Suitable monoclonal antibodies to IL-12R can be modified by known methods to provide chimeric, humanized or single chain antibody (SCA) embodiments.

The IL-12R antibodies of the present invention can be used to determine IL-12 receptor expression on human cells, such as peripheral blood lymphocytes and bone marrow cells, in normal and pathological conditions. The antisera and monoclonal antibodies of the invention can also be used to block IL-12 binding to its receptor and thus block its biologic activity. Neutralizing antibodies of the present invention can thus be used for therapeutic intervention in a number of disease states that are aggravated by activated T-cells and NK cells, such as autoimmune diseases, graft versus host disease and rheumatoid arthritis. Finally, as has been specifically demonstrated by the monoclonal antibody embodiment of the present invention, such antibody will also be useful for expression cloning strategies to isolate a cDNA coding for the IL-12 receptor.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 - Inhibiti n of ^{125}I-IL-12 Binding to IL-12 Receptor by Mouse Anti-IL-12R Antiserum

Ten fold serial dilutions of mouse anti-IL-12R immune serum (#211-1 and #211-2) and normal mouse serum (NMS) were preincubated with PHA-activated PBMC for 60 min at RT before addition of ^{125}I-IL-12 (100 pM). After addition of ^{125}I-IL-12, the reaction was incubated for 1-2 hrs at RT and the cell bound radioactivity was determined as outlined in "Methods". The data are expressed as the % Inhibition of ^{125}I-IL-12 binding in the presence of the immune serum when compared to the specific binding in the absence of serum.

Fig. 2 - Characterization of the IL-12 Binding Proteins on IL-12R Positive Human cells by Affinity-Crosslinking

PHA-activated PBMC (PHA-PBMC), Kit-225 (Kit-225) and K6 (K6) cells (1 x 10^7 cells/ml) were incubated with ^{125}I-IL-12 (100-500 pM) for 2 hrs at room temperature in the absence or presence of 25 nM unlabeled IL-12. Cells were then washed, affinity crosslinked with BS3 (0.4 mM final concentration) and a cell extract prepared as described in "Methods". The cell extract was precipitated with wheat germ lectin bound to solid supports as described in "Methods". The precipitated proteins were released by treatment with sample buffer and analyzed by SDS-PAGE and autoradiography on a 8.0% slab gel. The complex of ^{125}I-IL-12 crosslinked to the IL-12 receptor migrates as a single major band of approximately 210-250 kDa. The band migrating at 75 kDa is ^{125}I-IL-12 that was bound but not crosslinked to the IL-12 receptor. ^{125}I-IL-12 (IL-12) and ^{125}I-IL-12 that was treated with the BS3 crosslinker (IL-12/BS3) were electrophoresed in parallel lanes as markers for the migration of the 75 kDa IL-12 heterodimer and for any oligomers of IL-12 that may form with the BS3 crosslinker. The molecular sizes indicated in the margins were estimated from standards run in parallel lanes. Exposure time was 7 days.

Fig. 3 - Immunoprecipitation of the Solubilized ^{125}I-IL-12/IL-12R Crosslinked Complex by Anti-IL-12R Antibodies

Soluble complexes of ^{125}I-IL-12/IL-12R were prepared from PHA-activiated human PBMC as detailed in "Methods" and Figure 2. and immunoprecipitated by immobilized antibodies, 2*4E6, 2C6, 4D6, 20C2 and control. The soluble complexes were also precipitated with wheat germ lectin immobilized on crosslinked agarose (WG). The precipitated proteins were analyzed as described in "Methods'" and in Figure 2. Antibodies 4D6 and 20C2 are non-neutralizing and neutralizing anti-IL-12 antibodies, respectively. 4D6 immunoprecipitates ^{125}I-IL-12/IL-12R complex and free ^{125}I-IL-12, whereas 20C2 only immunoprecipitates free ^{125}I-IL-12. Both 2*4E6 and 2C6 recognize the ^{125}I-IL-12/IL-12R complex. ^{125}I-IL-12 (IL-12) and ^{125}I-IL-12 that was treated with the BS3 crosslinker (IL-12/BS3) were electrophoresed in parallel lanes as markers for the migration of the 75 kDa IL-12 heterodimer and for any oligomers of IL-12 that may form with the BS3 crosslinker. The molecular sizes indicated in the margins were estimated from standards run in parallel lanes. Exposure time was 7 days.

Fig. 4 - Equilibrium binding of ^{125}I-2*4E6 to PHA-activated PBMC at Room Temperature

Lymphoblasts (1 x 10^6 cells) were incubated for 2 hrs at room temperature with increasing concentrations of ^{125}I-2*4E6 in the absence (°) or presence (•) of 25 nM unlabeled 2*4E6. Total (°) and non-specific (•) cell bound radioactivity were determined as described in "Methods". Specific binding of ^{125}I-2*4E6 (∇) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data according to the method of Scatchard as determined by Ligand computer program with a single-site model.

Fig. 5 - Equilibrium Binding of ^{125}I-2*4E6 to Human K6 Cells at Ro m Temperature

K6 cells (1 x 10^6 cells) were incubated for 2 hrs at room temperature with increasing concentrations of ^{125}I-2*4E6 in the absence (•) or presence (∇) of 25 nM unlabeled 2*4E6. Total (•) and non-specific (∇) cell bound radioactivity were determined as described in "Methods". Specific binding of ^{125}I-2*4E6 (▼) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the biding data according to the method of Scatchard as determined by Ligand with a single-site model.

Fig. 6 - Inhibition of ^{125}I-2*4E Binding to K6 Cells by Purified 2*4E6 (24E6), Human IL-12 (HUIL-12) and Control Antibody (Control IgG)

The data are expressed as the amount of ^{125}I-2*4E6 bound [CPM BOUND (Percent)] to the cells in the presence of the indicated concentrations of unlabeled antibody or IL-12 when compared with the total specific binding in the absence of unlabeled competitor.

Fig. 7 - Equilibrium Binding of ^{125}I-IL-12 to Human K6 Cells at Room Temperature

K6 cells (1 x 10^6 cells) were incubated for 2 hrs at room temperature with increasing concentrations of ^{125}I-IL-12 in the absence (°) or presence (•) of 50 nM unlabeled IL-12. Total (°) and non-specific (•) cell bound radioa tivity were determined as described in Materials and Methods. Specific binding of ^{125}I-IL-12 (∇) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data according to the method of Scatchard as determined by Ligand with a single-site model.

Fig. 8 - Equilibrium Binding of ^{125}I-IL-12 t Detergent Solubilized IL-12R from K6 Cells

K6 cells 1.5 x 10^8 cells/ml) were solubilized with 8 mM CHAPS extraction buffer and the cell extract (0.2 ml) was immunoprecipitated for 16 hrs at 4°C with mAb 2*4E6 immobilized on goat anti-mouse IgG coupled to agarose as described in "Methods". Following this incubation, the beads were pelleted, washed and resuspended in IP buffer containing ^{125}I-IL-12 at concentrations ranging from 7 pM to 7.5 nM. The IL-12R immobilized on the 2*4E6 coated beads was incubated with ^{125}I-IL-12 for 2 hrs at RT and IL-12R bound radioactivity was determined in the presence of 50 nM unlabelled IL-12. The right hand panels show analysis of the binding data according to the method of Scatchard as determined by Ligand with a single-site model.

Fig. 9 - Western Blot Analysis of Detergent Solubilized IL-12R with mAb 2*4E6

PHA-activated PBMC (1 x 10^8 cells/ml) were solubilized with 8 mM CHAPS extraction buffer and the cell extract (1 ml) was immunoprecipitated as described in Figure 8. Following this incubation, the beads were pelleted, washed and the bound proteins released by treatment with 0.1 M glycine pH 2.3. The released proteins were separated by non-reducing SDS/PAGE on 8% gels transferred to nitrocellulose membrane and probed with ^{125}I-2*4E6 as described in "Methods". The molecular sizes indicated in the margins were estimated from molecular weight standards (Amersham Prestained High Molecular Weight Standards) run in parallel lanes. Exposure time was 7 days.

Fig. 10 - Equilibrium Binding of ^{125}I-IL-12 to Human Recombinant IL-12 Receptor Expressed in COS Cells

COS cells were transfected with a plasmid expressing human rIL-12R as described in "Methods". Three days later, transfected cells (1 x 10^4 cells) were incubated for 2 hrs. at room temperature with increasing

concentration f ^{125}I-IL-12 in the absence (°) or presence (□) of 50 nM unlabeled IL-12. Total (°) and non-specific (□) cell b und radi activity were determined as described in "Methods". Specific binding of ^{125}I-IL-12 (▲) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data according to the method of Scatchard as determined by Ligand with a single-site model.

Fig. 11 - Equilibrium Binding of ^{125}I-2*4E6 to Human Recombinant IL-12 Receptor Expressed in COS Cells.

COS cells were transfected with a plasmid expressing human rIL-12R as described in "Methods". Three days later, transfected cells (1 x 10^4 cells) were incubated for 2 hrs at room temperature with increasing concentrations of ^{125}I-2*4E6 in the absence (o) or presence (□) of 50 nM unlabeled 2*4E6. Total (o) and non-specific (□) cell bound radioactivity were determined as described in 'Methods". Specific binding of ^{125}I-2*4E6 (▲) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data according to the method of Scatchard as determined by Ligand with a single-site model.

Fig. 12 - Detection of IL-12 Receptor on Human Cells by Flow Cytometry

Day 4 PHA-activated lymphoblasts, Kit-225 and K6 cells were analyzed for IL-12R expressing cells by the indirect fluorescent antibody-labeling technique described in "Methods". The figure depicts specific staining for IL-12R obtained in the presence of mAb 2*4E6 (IL-12R) and non-specific staining obtained in the presence of a control antibody specific for IL-1 receptor (anti-Hu IL-1R), a control antibody specific for human IL-12 (4D6 + GART-PE CTRL) and the goat anti-mouse antibody conjugated with PE (GART-PE CTRL).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel antisera and monoclonal antibodies to the human IL-12 receptor. The antisera of the invention can be conveniently produced by immunizing host animals with PHA-activated human PBMC. Suitable host animals include rodents, such as, for example, mice, rats, rabbits, guinea pigs and the like, or higher mammals such as goats, sheep, horses and the like. Initial doses and booster shots can be given according to accepted protocols for eliciting immune responses in animals, e.g., in a preferred embodiment mice received an initial dose of 6 x 10^7 cells/mouse i.p. and five subsequent booster shots of between 2-5 x 10^7 cells over a six month period. Immunized mice were observed to develop an immune response against the human IL-12R as determined by inhibition of ^{125}I-IL-12 binding to PHA-activated PBMCs (Figure 1) and immunoprecipitation of the complex of ^{125}I-IL-12 crosslinked to IL-12R, which methods provide a convenient way to screen for hosts which are producing antisera having the desired activity.

Monoclonal antibodies are produced conveniently by immunizing Balb/c mice according to the above schedule followed by injecting the mice with 1x10^7 cells i.p. and 2.5 x 10^6 cells i.v. on two successive days starting four days prior to the cell fusion. Other protocols well known in the antibody art may of course be utilized as well.

B lymphocytes obtained from the spleen, peripheral blood, lymph nodes or other tissue of the host may be used as the monoclonal antibody producing cell. Most preferred are B lymphocytes obtained from the spleen. Hybridomas capable of generating the desired monoclonal antibodies of the invention are obtained by fusing such B lymphocytes with an immortal cell line, that is a cell line that which imparts long term tissue culture stability on the hybrid cell. In the preferred embodiment of the invention the immortal cell may be a lymphoblastoid cell or a plasmacytoma cell such as a myeloma cell, itself an antibody producing cell but also malignant. Murine hybridomas which produce IL-12R monoclonal antibodies are formed by the fusion of mouse myeloma cells and spleen cells from mice

immunized against hIL-12R expressed on the surface of activated peripheral bl od mononuclear cells. Chimeric and humanized monoclonal antibodies can be produced by cloning the antibody expressing genes from the hybridoma cells and employing recombinant DNA methods now well known in the art to either join the subsequence of the mouse variable region to human constant regions or to combine human framework regions with complementarity determining regions (CDR's) from a donor mouse or rat immunoglobulin. (See, for example, EPO Publication No. 0239400). An improved method for carrying out humanization of murine monoclonal antibodies which provides antibodies of enhanced affinities is set forth in International Patent Application No. WO 92/11018.

Polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities. These polypeptide fragments may be produced by proteolytic cleavage of intact antibodies by methods well known in the art, or by inserting stop codons at the desired locations in expression vectors containing the antibody genes using site-directed mutageneses to produce Fab fragments or $(Fab')_2$ fragments. Single chain antibodies may be produced by joining VL and VH regions with a DNA linker (see Huston et. al., Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird et. al., Science, 242, 423-426 (1988).

It is also within the skill of the art to utilize the monoclonal antibodies of the present invention as therapeutic agents. They may be formulated for parenteral administration in a manner known in the art such as by dissolving the purified monoclonal antibody product either intact or as a fragment in water for injection and sterile filtering. The dosage form may contain known excipients for parenteral administration of proteins such as buffers, stabilizers and carrier protein. The administered dosage will be selected by the attending physician by giving due consideration to the disease severity and nature as well as the age, size and condition of the patient. As immunoglobulins have demonstrated extended half-lifes in patients dosing every 10-14 days is usually sufficient. It is also within the skill of the art to modify the monoclonal antibody by forming a hybrid

with a toxin m lecule such as with a pseud monas exot xin or with the A chain of ricin to pr vide a hybrid molecule capable of destroying the cells expressing the IL-12R in a selective manner.

The invention also pertains to a method for detecting peripheral blood cells which express the IL-12 receptor, which comprises contacting a sample which contains the subject cells with substances capable of forming complexes with the IL-12 receptors so as to form cellular complexes between the substances and the IL-12 receptors, and detecting such cellular complexes. Another embodiment of the invention provides a method of evaluating cell activity in a subject which comprises detecting peripheral blood cells according to the method described above.

In the preferred embodiments, the substances are capable of forming complexes only with the IL-12 receptors present on the surface of peripheral blood cells in which the receptors were expressed. Particularly preferred are substances which comprise IL-12 monoclonal antibody.

One embodiment of the invention provides a method of evaluating immune cellular activity which comprises:

a. isolating peripheral blood mononuclear cells;
b. treating the cells with the IL-12 monoclonal antibody; and
c. determining the amount of monoclonal antibody bound to the cells.

The invention also involves a method for diagnosing an immune system abnormality in a subject which comprises determining the number of T cells, NK cells, or B-cells in a sample derived from the subject. This method involves contacting the sample with substances capable of forming complexes with the IL-12 receptors and determining the percentage of T cells, NK cells or B cells in the sample which have the IL-12 receptor. Comparing the percentages so determined with the percentage of cells which have the IL-12 receptor in a sample from a normal subject who does not have the immune system abnormality, the differences in the percentage of cells so determined being indicative of the immune system abnormality. Preferably, the subject is an animal, e.g., a human.

As a molecule associated with T cell, NK cell and B cell function, the measurement of IL-12R expression has diagnostic importance. Because IL-12R is distinctive t activated T cells, NK cells or B cells, it is a unique marker for these cells in a population of lymphocytes.

Moreover, the level of expression of IL-12R provides a measure of T cell, NK cell or B cell activity. This information may be important for evaluating the immune status of an individual. For instance, in the treating of certain disease, such as cancer, agents which affect the immunocompetency are often used. Assays for IL-12R expression may allow physicians to monitor the immune status of the patient and to adjust treatment to minimize the risk of opportunistic infection, often a threat to immunocompromised patients.

Assays for IL-12R expression may be conventional immunochemical assays for cell surface antigens. Peripheral blood mononuclear cells can be isolated from patient and incubated with IL-12R monoclonal antibody under conditions which allow the antibody to bind the surface antigen. Antibody bound to the cell surface provides a measure of IL-12R expression. Binding of the antibody to cells may be evaluated by employing an IL-12R monoclonal antibody labeled with a radioactive, fluorescent or other compound capable of being detected.

The invention also involves a method for detecting soluble IL-12 receptor concentration in samples derived from subjects with immune system disorders, cancer, or other diseases that would be marked by an increase or decrease in soluble form of IL-12R. Assays for soluble IL-12R may be conventional sandwich immunochemical assays or ^{125}I-IL-12 binding assays to immobilized IL-12R.

Certain embodiments of this invention are exemplified in the Examples and Experimental Discussion which follow. In these sections, possible mechanisms and structures are postulated. The Examples and the Experimental Discussion are set forth to aid in an understanding of the

invention but are not intended to, and should not be construed t , limit in any way the invention as set forth in the claims which follow.

Example 1

Preparation, Characterization & Purification of Hybridoma Antibodies

Balb/c mice (Charles River Laboratories) were immunized by the intraperitoneal route with PHA-activated human PBMC (PHA-activated PBMC) at 6 x 10^7 cells/mouse. Mice received 5 subsequent booster injections of between 2-5 x 10^7 cells over a six month period. For preparation of activated spleen cells, 2 mice were injected intraperitoneally and intravenously with 1 x 10^7 and 2.5 x 10^6 cells, respectively, on two successive days, starting four days prior to the cell fusion. Spleen cells were isolated from these mice and fused with SP2/0 cells at a ratio of 1:1 with 35% v/v polyethylene glycol 4000 (E. Merck) according to the method of Fazekas et al., J. Immunol. Methods 35, 1 (1980). The fused cells were plated at a density of 6 x 10^5 cells/ml/well in 48-well cluster dishes in IMDM supplemented with 10% FBS, glutamine (2mM), β-mercaptoethanol (0.1mM), gentamicin (50g/ml), 5% ORIGEN hybridoma cloning factor (IGEN, Inc.), 5% P388D1 supernatant (10) and 100 Units/ml rHuIL-6. Hybridoma supernatants were assayed for specific anti-IL-12 receptor antibodies by: 1) immunoprecipitation of the soluble complex of ^{125}I-HuIL-12 crosslinked to IL-12 receptor (125I-IL-12/IL-12R), 2) inhibition of ^{125}I-HuIL-12 binding to PHA-activated PBMC's, and 3) differential binding to IL-12 receptor positive cells verses receptor negative cells). Hybridoma cell lines secreting specific anti-receptor antibodies were cloned by limiting dilution. Antibodies were purified from ascites fluids by affinity chromatography on Protein G bound to cross-linked agarose according to the manufacturer's protocol (Genex).

Example 2

Preparati n f Human PHA Lymph blasts and IL-12 Receptor Binding Assays

Human peripheral blood mononuclear cells were isolated (see Gately et al. J. Natl. Cancer Inst. 69, 1245 (1982)) and cultured at 37°C at a density of 5 x 105 cells/ml in (tissue culture medium (TCM) containing 0.1% PHA-P (Difco). After 3 days, the cultures are split 1:1 with fresh TCM, and human rIL-2 was added to each culture to give a final concentration of 50 units/ml. The cultures were then incubated for an additional 1-2 days, prior to use in assays.

PHA-activated human PBMC were washed once in binding buffer (RPMI-1640, 5% FBS, 25 mM HEPES pH 7.4) and resuspended in binding buffer to a cell density of 7 X 10^6 cells/ml. Lymphoblasts (7 X 10^5 cells) were incubated with various concentrations of ^{125}I-IL-12 (5-10000 pM) at room temperature for the designated times. Cell bound radioactivity was separated from free ^{125}I-IL-12 by centrifugation of the assay mixture through 0.1 ml of an oil mixture (1:2 mixture of Thomas Silicone Fluid 6428-R15 : A.H. Thomas, and Silicone Oil AR 200 : Gallard-Schlessinger) at 4°C for 90 sec at 10,000 X g. The tip containing the cell pellet was excised, and cell bound radioactivity was determined in a gamma counter. Non-specific binding was determined by inclusion of 100 nM unlabeled IL-12 in the assay. Incubations were carried out in duplicate or triplicate. Receptor binding data were analyzed by using the non-linear regression programs EBDA and LIGAND as adapted for the IBM personal computer by McPherson, J. Pharmacol Methods 14, 213 (1985) from Elsevier-BIOSOFT.

Example 3

Affinity Cross-Linking of ^{125}I-IL-12 to IL-12 Receptor Bearing Cell Lines

IL-12 receptor bearing cells were incubated with ^{125}I-IL-12 (100-500 pM) in the presence or absence of excess unlabeled IL-12 for 2 hr at room

temperature. The cells w re then washed with ice-cold PBS pH 8.3 (25mM Sodium Phosphate pH 8.3, 0.15 MNaCL and 1mM MgCl2) and resuspend d at a concentration of 0.5-1.0x10^7 cells/ml in PBS pH 8.3. BS3 (Pierce) in dimethyl sulfoxide was added to a final concentration of 0.4 mM. Incubation was continued for 30 min. at 4°C with constant agitation. The cells were washed with ice-cold 25 mM Tris-HCl (pH 7.5), 0.15 m NaCl and 5 mM EDTA and then solublized at 0.5 - 1.0 x 10^8 cells/ml in solubilization buffer (50 mM Tris-HCl (pH 8.0) containing 8mM CHAPS, 0.25 M NaCl, 5mM EDTA, 40 µg/ml PMSF, 0.05% NaN3, and 1% BSA) for 1 hr at 4°C. The extracts were centrifuged at 12,000 x g for 45 min. at 4°C to remove nuclei and other debris.

Example 4

Immunoprecipitation Assay of the Soluble Complex of ^{125}I-IL-12 Crosslinked to Human IL-12R.

For the immunoprecipitation assay, hybridoma culture supernatant (0.5 ml), diluted antisera, or purified IgG was added to a microfuge tube containing 0.1 ml of a 50% suspension of either goat-anti-mouse IgG coupled to agarose (SIGMA CHEM. CO.) or Protein G coupled to Sepharose 4B (Pharmacia). The assay volume was brought up to 1.0 ml with IP buffer (8 mM CHAPS in PBS (.25 MNaCl), 1% BSA, & 5 mM EDTA) and the mixture was incubated on a rotating mixer for 2 hr at room temperature. The beads were pelleted by centrifugation, resuspended in 1 ml IP buffer containing ^{125}I-IL-12/IL-12R (10-20,000 cpm) and the mixture was incubated on a rotating mixer for 16 hr at 4°C. After this incubation, the beads were pelleted by centrifugation and washed twice in IP buffer without BSA. The ^{125}I-labelled receptor complex bound to the solid phase antibodies was released by adding 100 µl of 2x Laemmli sample buffer (Nature 227, 680 (1970)) with and without 10%-mercaptoethanol and heating for 5 min. at 95°C. The immunoprecipitated proteins were analyzed by SDS-PAGE on 8% or 4-15% gradient polyacrylamide gels and visualized by autoradiography.

Example 5

Assays for IL-12R Solubilized fr m Cells Expressing IL-12 Recept r.

To confirm that the antibodies identified by the immunoprecipitation assay were specific for IL-12R, an immunoprecipitation/soluble IL-12R binding assay was developed. As described in Example 1 above, antibodies (as hybridoma supernatant, purified IgG (50 µg) or antisera) were immobilized by binding to goat anti-mouse IgG coupled to agarose (100 µl; Sigma Chemical Co.) or protein G coupled to Sepharose 4B (100 µl; Pharmacia). For some experiments, antibodies were covalently crosslinked to protein G-Sepharose 4B, before being used in the assay (See Stern and Podlaski, Techniques in Protein Chemistry (1993). The immobilized antibodies were resuspended in IP buffer (0.3 ml) and 0.2 ml of a detergent solubilized extract of PHA-activated PBMCs or K6 cells that contained IL-12R was added. To prepare the detergent solubilized IL-12R preparation, the cells were washed with ice-cold 25 mM Tris-HCl (pH 7.5), 0.15 M NaCl and 5 mM EDTA and then solublized at 1.5×10^8 cells/ml in solubilization buffer (50 mM Tris-HCl, pH 8.0, containing 8mM CHAPS, 0.25 M NaCl, 5mM EDTA, 40 µg/ml PMSF, 0.05% NaN3, and 1% BSA) for 1 hr at 4°C. The extracts were centrifuged at 120,000 x g for 60 min. at 4°C to remove nuclei and other debris. The mixture was incubated on a rotating mixer for 16 hr at 4°C. After this incubation, the beads were pelleted by centrifugation and resuspended in IP buffer (0.15 ml) containing ^{125}I-HuIL-12 at concentrations ranging from 0.05 to 7.5 nM. The IL-12R immobilized on the antibody coated beads was incubated with ^{125}I-HuIL-12 for 2 hrs. at room temperature on a shaker. Following this incubation, the beads were pelleted, washed twice with IP buffer and the bound radioactivity determined in a gamma counter. Nonspecific binding was determined by inclusion of 70 nM unlabeled human IL-12 in the assay. Solubilized IL-12R binding data were analyzed according to the method of Scatchard, (Assn. N.Y. Acad. Sci. 51, 660 (1949)) by using the nonlinear regression programs EBDA and Ligand as adapted for the IBM PC by McPherson, supra from Elsevier-BIOSOFT.

Example 6

Competitive Inhibiti n f ^{125}I-IL-12 Receptor Binding by Antibodies

The ability of hybridoma supernatant solutions, purified IgG, or antisera to inhibit the binding of ^{125}I-IL-12 to PHA-activated lymphoblasts was measured as follows: serial dilutions of culture supernatants, purified IgG or antisera were mixed with activated lymphoblasts (1-1.5 x 10^6 cells) in binding buffer (RPMI-1640, 5% FBS + 25 mM Hepes pH 7.4) and incubated on an orbital shaker for 1 hour at room temperature. ^{125}I-HuIL-12 (1 x 10^5 cpm) was added to each tube and incubated for 1-2 hours at room temperature. Non-specific binding was determined by inclusion of 10 nM unlabeled IL-12 in the assay. Incubations were carried out in duplicate or triplicate. Cell bound radioactivity was separated from free ^{125}I-IL-12 by centrifugation of the assay through 0.1 ml of an oil mixture as described above. The tip containing the cell pellet was excised, and cell bound radioactivity was determined in a gamma counter.

Example 7

Labeling of Human IL-12 and Mab 2*4E6 with ^{125}I.

Human IL-12 and purified 2*4E6 IgG were labelled with ^{125}I by a modification of the Iodogen method (Pierce Chemical Co., Rockford, IL). Iodogen was dissolved in chloroform and 0.05 mg dried in a 12 x 15 mm borosilicate glass tube. For radiolabeling, 1.0 mCi Na[^{125}I] (Amersham, Chicago, IL) was added to an Iodogen-coated tube containing 0.05 ml of Tris-iodination buffer (25 mM Tris-HCL pH 7.5, 0.4 M NaCl and 1 mM EDTA) and incubated for 4 min at room temperature. The activated 125I solution was transferred to a tube containing 0.05 to 0.1 ml IL-12 (7 µg) or IgG (100 µg) in Tris-iodination buffer and the reaction was incubated for 9 min at room temperature. At the end of the incubation, 0.05 ml of Iodogen stop buffer (10 mg/ml tyrosine 10% glycerol in Dulbecco's PBS, pH 7.40) was added and reacted for 3 min. The mixture was then diluted with 1.0 ml Tris-iodination buffer, and applied to a Bio-Gel P10DG desalting column

(BioRad Laboratories) for chr matography. The column was eluted with Tris-iodination buffer, and fractions (1 ml) containing the peak amounts of labelled protein were combined and diluted to 1 x 108 cpm/ml with 1% BSA in Tris-iodinati n buffer. The TCA preciptable radioactivity (10% TCA final concentration) was typically in excess of 95% of the total radioactivity. The radiospecific activity was typically ~ 1500 to 2500 cmp/fmol for 2*4E6 IgG and 5000 to 7000 cpm/fmole for IL-12.

Example 8

Binding Assays of ^{125}I-2*4E6 to Intact Cells.

PHA-activated human PBMC were washed once in binding buffer (RPMI 1640, 5% FBS and 25 mM Hepes, pH 7.4) and resuspended in binding buffer to a cell density of 1.5 x 10^7 cells/ml. Lymphoblasts (1.5 x 106 cells) were incubated with various concentrations of ^{125}I-2*4E6-IgG (.005 to 2 nM) at room temperature for 1.5 hrs. Cell bound radioactivity was separated from free ^{125}I-2*4E6 IgG by centrifugation of the assay mixture through 0.1 ml silicone oil at 4°C for 90 seconds at 10,000 x g. The tip containing the cell pellet was exercised, and cell bound radioactivity was determined in a gamma counter. Non-specific binding was determined by inclusion of 67 nM unlabeled 2*4E6 IgG in the assay. Incubations were carried out in duplicate or triplicate. Receptor binding data were analyzed by using the nonlinear regression programs EBDA, Ligand and Kinetics as adapted for the IBM personal computer by McPherson, supra from Elsevier BIOSOFT.

Example 9

Expression of Recombinant IL-12R in COS Cells and Determination of ^{125}I-2*4E6 Binding.

COS cells (4-5 X 10^7) were transfected by electroporation with 25 µg of plasmid DNA expressing recombinant human IL-12R (U. Gubler and A. Chua, unpublished observations) in a BioRad Gene Pulser (250 µF, 250 volts) according to the manufacturer's protocol. The cells were plated in a

600 cm2 culture plate, harvested after 72 hours by scraping, washed and resuspended in binding buffer. Transfected cells (8 X 10^4 were incubated with increasing c ncentrations of ^{125}I-labeled 2*4E6 or IL-12 at room temperature for 2 hrs. Cell bound radioactivity was separated from free ^{125}I-labeled 2*4E6 or IL-12 as described above.

Example 10

Western Blot Analysis of Soluble IL-12R with mAb 2*4E6

PHA-activated PBMC were washed 3 times with ice-cold PBS and solubilized at 0.5 - 1 x 10^8 cells/ml in solubilization buffer (50 mM Tris-HCl pH 8.0 containing 8 mM CHAPS, 0.25 M NaCl, 5 mM EDTA, 40 µg/ml PMSF, .05% NaN3 and 1 mg/ml BSA) for 1 hr at 4°C. The extracts were centrifuged at 12,000 x g for 45 min. at 4°C to remove nuclei and other debris. The extracts were incubated with 2*4E6 IgG or control IgG bound to goat-anti-mouse IgG immobilized on cross-linked agarose (Sigma Chemical Co.). The precipitated proteins were released by treatment with 0.1 M glycine pH 2.3, neutralized with 3M Tris, mixed with 1/5 volume of 5 x Laemmli sample buffer, and separated by SDS/PAGE on 8% pre-cast acyrlamide gels (NOVEX). The separated proteins were transferred to nitrocellulose membrane (0.2 µM) for 16 hours at 100 volts in 10 mM TRIS-HCL (pH 8.3), 76.8 mM glycine, 20% methanol and 0.01% SDS. The nitrocellulose membrane was blocked with BLOTTO (50% w/v nonfat dry milk in PBS + .05% Tween 20) and duplicate blots were probed with ^{125}I-2*4E6 IgG (1 x 106 cpm/ml in 8mM CHAPS in PBS, 0.25 M NaCl, 10% BSA and 5 mM EDTA) + unlabelled 2*4E6 IgG (67nM).

Example 11

Analysis of IL-12 Receptor Expression on Human Cells by Fluorescence Activated Cell Sorting with mAb 2*4E6

To stain cells expressing IL-12 receptor, 1 x 10^6 in 100 µl staining buffer (PBS containing 2% FBS and 0.1% NaN3) were incubated with 10 µl of 2*4E6 ascites fluid for 25 min. at 4°C. Cells were then washed twice with staining

buffer followed by incubation with a 1:100 dilution of g at F(ab)2 anti mouse Ig-PE (Tag , Burlingame CA) for 25 min. at 4°C. The stained cells were washed twice with staining buffer and then analyzed on a FACScan flow cytometer (Beckton Dickinson).

Example 12

Inhibition of IL-12 Binding to Human PHA-Lymphoblasts by Mouse Anti-IL-12R Antiserum.

Mice immunized with PHA-activated PBMCs developed an immune response against the human IL-12R as determined by inhibition of ^{125}I-IL-12 binding to PHA-activated PBMCs (Figure 1) and immunoprecipitation of the complex of ^{125}I-IL-12 crosslinked to IL-12R (data not shown). The dilutions for half-maximal inhibition of ^{125}I-IL-12 binding to PHA-activated PBMCs were 1/500 and 1/250 for animals 211-1 and 211-2, respectively (Figure 1). These antisera also neutralized IL-12 biologic activity as measured in a PHA-lymphoblast proliferation assay (data not shown). Spleen cells isolated from these mice were fused with SP2/0 myeloma cells and the resulting hybridomas were initially screened for IL-12R specific antibodies by immunoprecipitation of the ^{125}I-IL-12/IL-12R complex and by inhibition of ^{125}I-IL-12 binding to IL-12R.

Example 13

Identification and Characterization of Monoclonal Anti-Il-12R Antibodies.

The immunoprecipitation assay identified 13 hybridomas secreting putative non-neutralizing anti-IL-12R antibodies, whereas the IL-12R binding assay identified 3 putative neutralizing IL-12R antibodies (Table 1). The immunoprecipitation assay measured the ability of the putative anti-IL-12R antibodies that are immobilized on a solid phase to capture the solubilized complex of ^{125}I-IL-12/IL-12R. To verify that the radioactivity immunoprecipitated by the immobilized antibody was present in the complex of ^{125}I-IL-12/IL-12R, the immunoprecipitated

proteins were solubilized, separated by SDS-PAGE and visulaized by autoradi graphy. The preparations of the ^{125}I-IL-12/IL-12R complexes s lubilized from PHA-activated PBMC, Kit-225 and K6 cells were resolved into tw major radioactive bands, 210-250 kDa and 75 kDa (Figure 2). The 210-250 kDa and 75 kDa complexes were identified as the ^{125}I-IL-12/IL-12R complex and ^{125}I-IL-12 not complexed with the receptor, respectively (Figure 2). See also Chizzonite et al., J. Immunol. <u>148</u>, 3117 (1992). The radioactive 75 kDa band visualized from the cell extracts co-migrated with ^{125}I-IL-12, indicating that it represented ^{125}I-IL-12 that bound but was not covalently crosslinked to IL-12R. The 210-250 kDa band was not a covalent crosslinked oligomer of ^{125}I-IL-12 because it is not produced when the crosslinking agent BS3 was added directly to ^{125}I-IL-12 (Figure 2).

Hybridoma cells secreting putative anti-IL-12R antibodies were then cloned by limiting dilution and screened by both the immunoprecipitation and inhibition of binding assays that identify non-nuetralizing and neutralizing antibodies, respectively. During this cloning and screening process, hybridoma lines secreting putative neutralizing anti-IL-12R antibodies were not recovered, whereas non-neutralizing antibodies were recovered from both the original immunoprecipitation and inhibitory positive hybridomas. After this initial identification and cloning, a direct binding assay was used to determine if the non-neutralizing antibodies only bound to cells expressing IL-12R. This assay demonstrated that the non-neutralizing antibodies could be divided into 2 classes, those that bound only IL-12R postive human cells and those that bound to most human cells (data not shown). Representitive antibodies from each class, 2*4E6 and 2C6, respectively, were produced in ascites fluid, purified by protein G affinity chromatography and extensively characterized.

Table 1: INITIAL IDENTIFICATION OF HYBRIDOMAS SECRETING ANTI-IL-12 RECEPTOR ANTIBODIES: SPLENOCYTES FROM MICE #211-1 AND #211-2

HYBRIDOMA/ANTIBODY		I.P. ASSAY[1] (cpm bound)	INHIBITION ASSAY[2]
IL-12R 2C6[3]		1900	-
211-1	1A5	722	-
	4E6	840	-
	5C1	312	+
211-2	3B1	1323	-
	4A3	2172	-
	4D6	804	-
	5D5	877	-
	4A5	509	+
	4C6	456	+
	1D1	1395	-
	5E6	2043	-
	2*4E6	2836	-
Control mAb		402	-

1 I.P. assay measures the amount of ^{125}I-IL-12/IL-12R complex bound by the immobilized antibody.

2 Inhibition assay measures whether the antibody can inhibit ^{125}I-IL-12 binding to PHA-activated PBMC.

3 IL-12R 2C6 is an antibody that both immunoprecipitates the ^{125}I-IL-12/IL-12R complex and binds to many IL-12R positive and negative human cells. This antibody probably recognizes a component closely associated with the IL-12R.

Example 14

Characteristics of Monclonal Anti-IL-12R Antibody 2*4E6 Binding to Natural IL-12R

MAb 2*4E6 immunoprecipitates the ^{125}I-IL-12/IL-12R complex solubilizied from PHA-activated human lymphoblasts, Kit-225 and K6 cells (Figure 3, data shown for PHA-activated PBMC), but does not block ^{125}I-IL-12 binding to IL-12R expressed on these cells. These data suggested that the 2*4E6 antibody was a non-inhibitory or non-neutralizing anti-IL-12R antibody. To confirm that 2*4E6 was an non-inhibitory antibody specific for the IL-12R, 2*4E6 was labeiled with ^{125}I and direct binding assays were performed with IL-12R positive and negative cells. ^{125}I-2*4E6 binds to IL-12R bearing cells with an affinity that ranges from 337 pM to 904 pM and identifies between 1500 and 5000 binding sites per cell (PHA-activated PBMC, Figure 4; K6 cells, Figure 5). IL-12 does not block ^{125}I-2*4E6 from binding to PHA-activated PBMCs and confirms that 2*4E6 is a non-inhibitory/non-neutralizing antibody (Figure 6). ^{125}I-2*4E6 binds to other cells expressing IL-12R, such as Kit 225, and YT cells, but does not bind to IL-12R negative cells (non-activated human PBMC, MRC-5 fibroblasts and HL-60 cells (Table 2).

Equilibrium binding assays have demonstrated that ^{125}I-IL-12 identifies 3 separate binding sites on the surface of PHA-activated PBMCs, Kit-225 and K6 cells (Figure 7, data for K6 cells and Table 2). Analysis of this binding data by the method of Scatchard, supra shows these affinities are approximately 5-20 pM, 50-200 pM and 2-6 nM, respectively. The total number of ^{125}I-IL-12 binding sites per cell are approximately 1500 to 5000, which is in good agreement with the total number of binding sites identified by ^{125}I-2*4E6 (Table 2). The data also suggests that 2*4E6 recognizes the low affinity (2-5 nM) binding component of the IL-12 receptor in much the same manner that the anti-TAC antibody recognizes the low affinity component (p55 subunit) of the IL-2 receptor.

Since the data indicat d that mAb 2*4E6 was a non-neutralizing antibody specific for the IL-12R, th molecular weight and ^{125}I-IL-12 binding characteristics of th protein(s) immunoprecipitated by mAb 2*4E6 from the surface of IL-12R postive cells was investigated. The steady state binding f ^{125}I-IL-12 t proteins immunoprecipitated by immobilized 2*4E6 from solubilized extracts of PHA-activated PBMCs, Kit-225 and K6 cells was saturable and specific (Figure 8, data for extracts from K6 cells). Transformation of the binding data by the method of Scatchard, revealed a single site with an apparent affinity of 188 pM. The proteins immunoprecipitated by 2*4E6 from the cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with ^{125}I-2*4E6 in a western blot. On the western blot, ^{125}I-2*4E6 binds to an approximately 90 kDa protein, that is only immunoprecipitated by 2*4E6 and not by an anti-IL-12 antibody or a control antibody (Figure 9, data shown for PHA-activated PBMCs). In summary, all the data demonstrated that mAb 2*4E6 bound a protein on the surface of IL-12R positive cells that was approximately 90 kDa and bound ^{125}I-IL-12 in a specific manner.

TABLE 2: COMPARISON OF THE BINDING OF IL-12 AND 2•4E6 TO HUMAN CELLS EXPRESSING IL-12 RECEPTOR.

Cell Type	IL-12 Binding[1]		2•4E6 Binding[2]	
	K_D (nM)	Sites/cell	K_D (nM)	Sites/cell
Human Cells				
non-activated human PBMC[3]	none detected		none detected	
PHA-PBMC (5-7 day) (3 sites)	0.018 0.084 1.800	312 501 1406	0.745	1472 - 2246
K6 cells (3 sites)	0.016 0.057 2.400	707 939 4036	0.489	3116 - 5259
Kit-225 (3 sites)	0.023 0.210 2.360	100 250 755	0.594	1950
YT cells (2 sites)	0.006 0.109	24 117	0.904	4522
RAJI cells	none detectable		0.450	561
MRC-5	none detectable		none detectable	
HL-60	none detectable		none detectable	

1 Steady state ^{125}I-IL-12 binding assays. Apparent dissociation constant (K_D) and binding sites per cell have been calculated by transformation of the data by the method of Scatchard (15).

2 Steady state ^{125}I-2•4E6 binding assays. Data transformed by the method of Scatchard (15).

3 Human peripheral blood mononuclear cells (PBMC) were activated with PHA as described in the methods (PHA-PBMC).

Example 15

MAb 2*4E6 Binding T Human Recombinant IL-12R Expressed in COS Cells.

The characteristics of the protein bound by mAb 2*4E6 fullfilled standard criterion for an IL-12R and therefore 2*4E6 was used in an expression cloning strategy to isolate a cDNA coding for the human IL-12R. A cDNA coding for the human IL-12R was isolated by this method (U. Gubler and A.O. Chua, unpublished observations). The IL-12R cDNA was engineered in a mammalian cell expression vector, transfected into COS-7 cells and the specificity for binding of ^{125}I-IL-12 and ^{125}I-2*4E6 was determined. Steady state binding of ^{125}I-IL-12 to the rIL-12R expressing COS cells identifies a single binding site with an apparent affinity of 2-3 nM and approximately 150,000 sites/cell (Figure 10). This low affinity IL-12 binding site corresponds to the low affinity site seen in the binding assays with human cells that naturally express IL-12R. The binding of 125I-2*4E6 to rIL-12R expressed in the COS cells is saturable and specific and identifies approximately 200,000 sites/cell (Figure 11). COS cells transfected with an unrelated plasmid do not bind either ^{125}I-IL-12 or ^{125}I-2*4E6 (data not shown). These data demonstrated unequivocally that mAb 2*4E6 was specific for the low affinity component of the IL-12R.

Example 16

Analsis of mAb 2*4E6 Binding to IL-12R Positive Human Cells by Fluorescence Acitvated Cell Sorting (FACS).

The expression level of IL-12R on human cells could be regulated depending on the activation state of the cells , the cell cycle or the type of environment from which the cells are isolated. Previous data had demonstrated that PHA activation of PBMC leads to a gradual rise in IL-12R expression, reaching a maximum at 3-4 days after activation and decling thereafter. Desai et al., J. Immunol. Methods 148, 3125 (1992) To investigate the heterogenity of IL-12R expression on PHA-activated

PBMCs, Kit-225 and K6 cells, FACS analysis of IL-12R on these cells was determined with mAb 2*4E6 (Figure 12). The fluorescence intensity of binding f 2*4E6 was specific and indicated that these three cell types expressed appr ximately equal numbers f IL-12R. Interestingly, the FACS analysis indicated that the cell population was fairly homogenous and did not have one population expressing no or low numbers of IL-12R and a second population that expressed very high numbers of IL-12R.

We claim:

1. An immunoglobulin capable of binding selectively to the human IL-12 receptor.

2. The immunoglobulin of claim 1, which is in the form of an anti-sera composition.

3. The immunoglobulin of claim 2 which is capable of neutralizing human IL-12 bioactivity by binding to human IL-12R.

4. The immunoglobulin of claim 2 which is capable of inhibiting the binding of human IL-12 to the human IL-12 receptor.

5. The immunoglobulin of claim 2 which is of murine origin.

6. The immunoglobulin of claim 1 which is in the form of a monoclonal antibody.

7. The immunoglobulin of claim 6 wherein said monoclonal antibody is in humanized form.

8. The immunoglobulin of claim 6 which is in the form of a single chain antibody.

9. A method for the detection of cell lines expressing the human IL-12 receptor which method comprises selectively binding said cells with an immunoglobulin comprising a monoclonal antibody capable of specifically binding to the IL-12 receptor and detecting such binding.

10. The method of claim 9 wherein the monoclonal antibody to the IL-12 receptor is covalently bound to a solid resin.

11. The method of claim 9 wherein said immunoglobulin is labeled with a detectable label.

12. The method of claim 11 wherein said detectable label is ^{125}I.

13. An assay for the detecti n of soluble IL-12 recept r which assay comprises capturing said receptor with an immunoglobulin capable of selectively binding to the IL-12 receptor and carrying out a binding assay with ^{125}I-labelled IL-12.

14. A composition comprising human cells activated to express human IL-12 receptor bound with an immunoglobulin capable of specifically binding to the human IL-12 receptor, which immunoglobulin is labeled with a detectable label.

ABSTRACT

This discl sure relates to novel antibodies specific t the recently discovered receptor to human interleukin 12 (hIL-12r). The antibodies to hIL-12r, most preferably, the monoclonal antibodies to that protein, are useful in determining the status of the human immune system and as diagnostic reagents or potential therapeutic reagents for conditions involving imbalances in IL-12 levels or cell types sensitive to IL-12 activation.

Further aspects of the disclosure relate to methods of producing and purifying such novel antibodies and hybridoma cell lines capable of their production. Another aspect of the disclosure relates to an immunoprecipitation assay for the detection of solubilized IL-12R which employs, in a preferred embodiment, monoclonal antibodies to the receptor of the present invention covalently bound to Protein G-Sepharose resin.

Attorney Docket No. CD, 8805

Declarati n and Power of Attorney for Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ANTIBODY TO IL-12 RECEPTOR

the specification of which

(check one)

[X] is attached hereto.

[ ] was filed on ______________________________ as

Application Serial No. ______________________________

and was amended on ______________________________
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
(Number)	(Country)	(Day/Month/Year Filed)	[ ] Yes	[ ] No
(Number)	(Country)	(Day/Month/Year Filed)	[ ] Yes	[ ] No
(Number)	(Country)	(Day/Month/Year Filed)	[ ] Yes	[ ] No

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the clams of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) (Filing Date) (Status) (patented, pending, abandoned)

(Application Serial No.) (Filing Date) (Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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Residence

Citizenship

Post Office Address

Full name of fourth joint inventor, if any

Fourth Inventor's signature
Date

Residence

Citizenship

Post Office Address

Full name of fifth joint inventor, if any

Fifth Inventor's signature Date

Residence

Citizenship

Post Office Address

Title 37, Code of Federal Regulations, §1.56
Duty to disclose information material to patentability.

(a) Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie of unpatentability of a claim: or

(2) It refuses, or is inconsistent with, a position the applicant takes in:
(i) Opposing an argument of unpatentability relied on by the Office, or
(ii) Asserting an argument of patentability.

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FIGURE 1

INHIBITION OF [125]-I-IL-12 RECEPTOR BINDING BY
MOUSE ANTI IL-12 RECEPTOR ANTISERUM

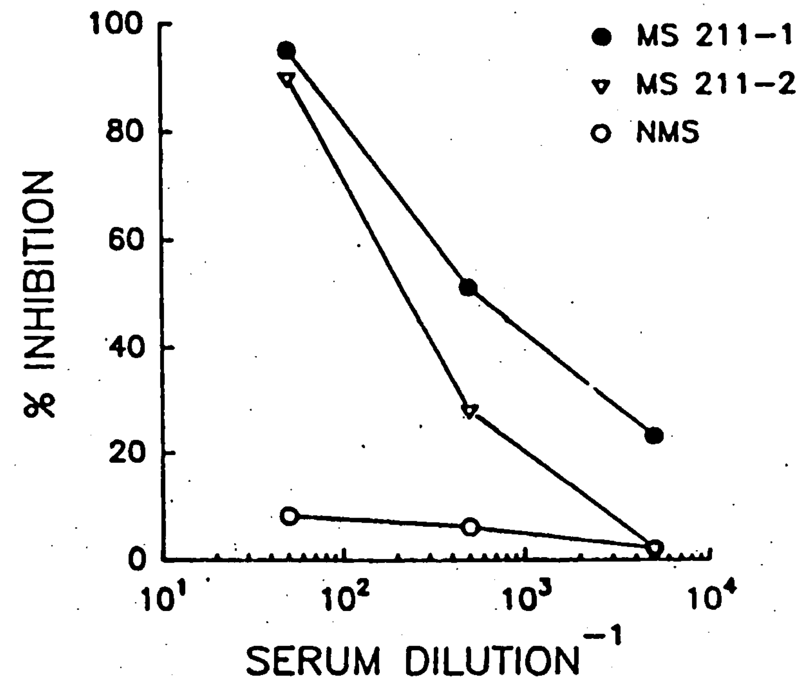

FIGURE 2

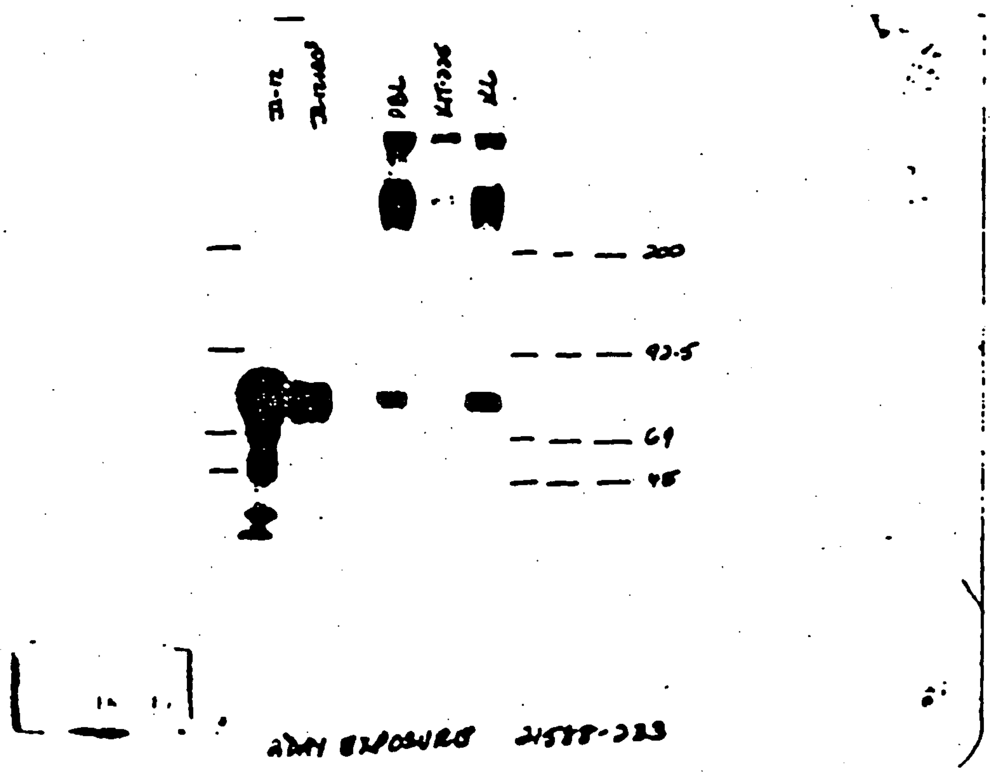

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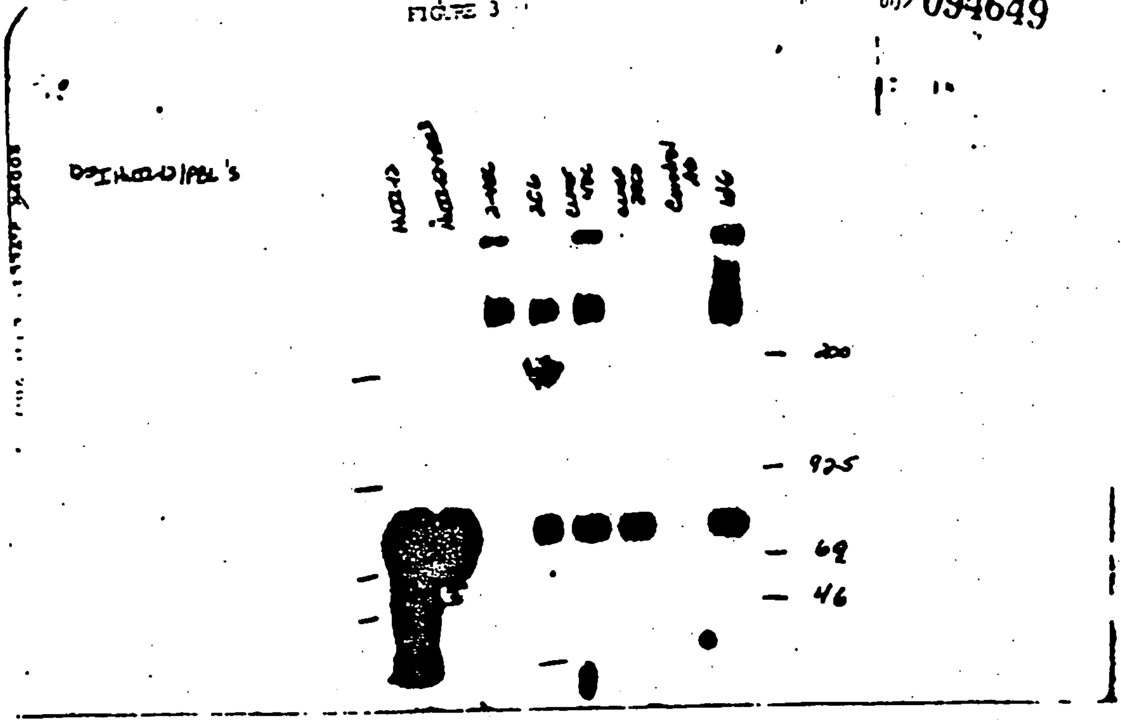
FIGURE 3



PHA-PBLS: [125]-I-24E6 IgG BINDING

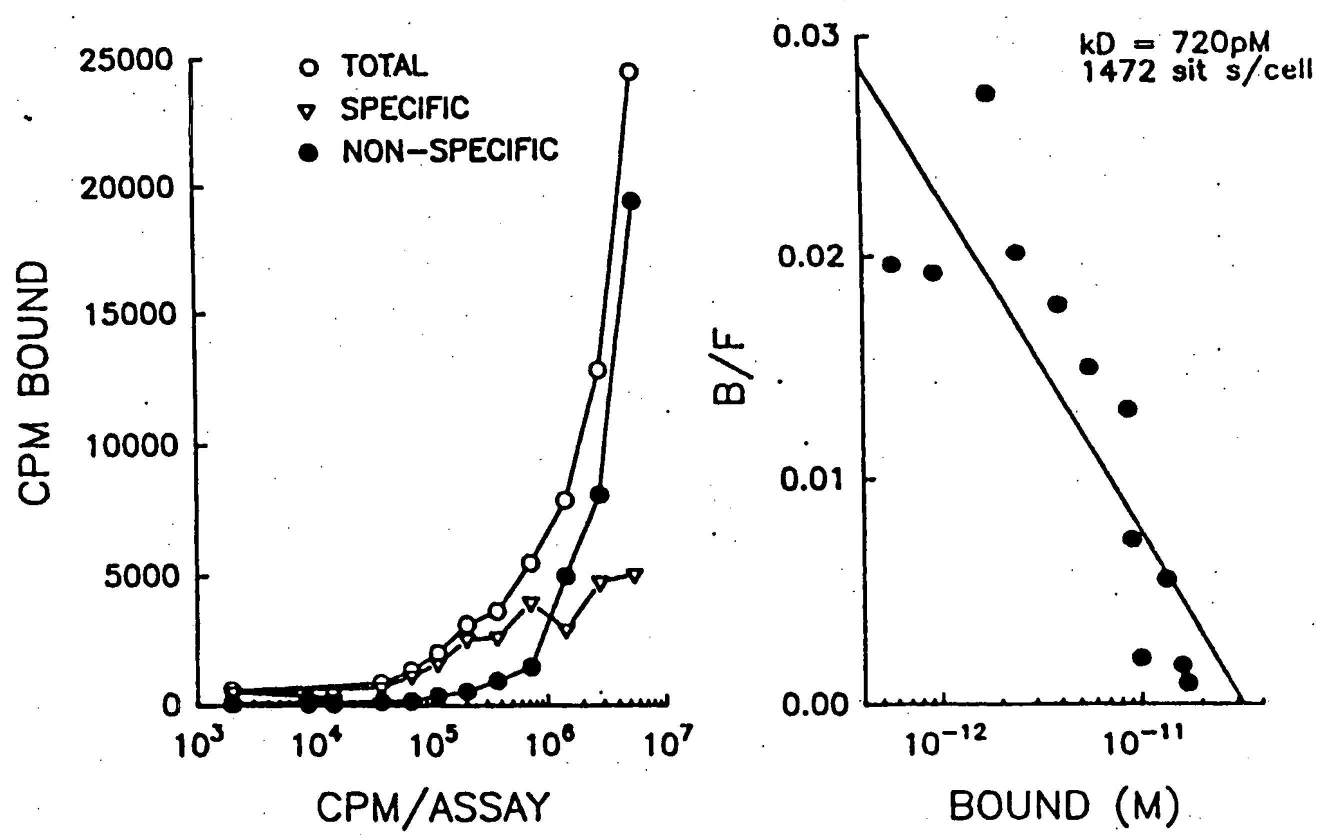


FIGURE 4

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FIGURE 5

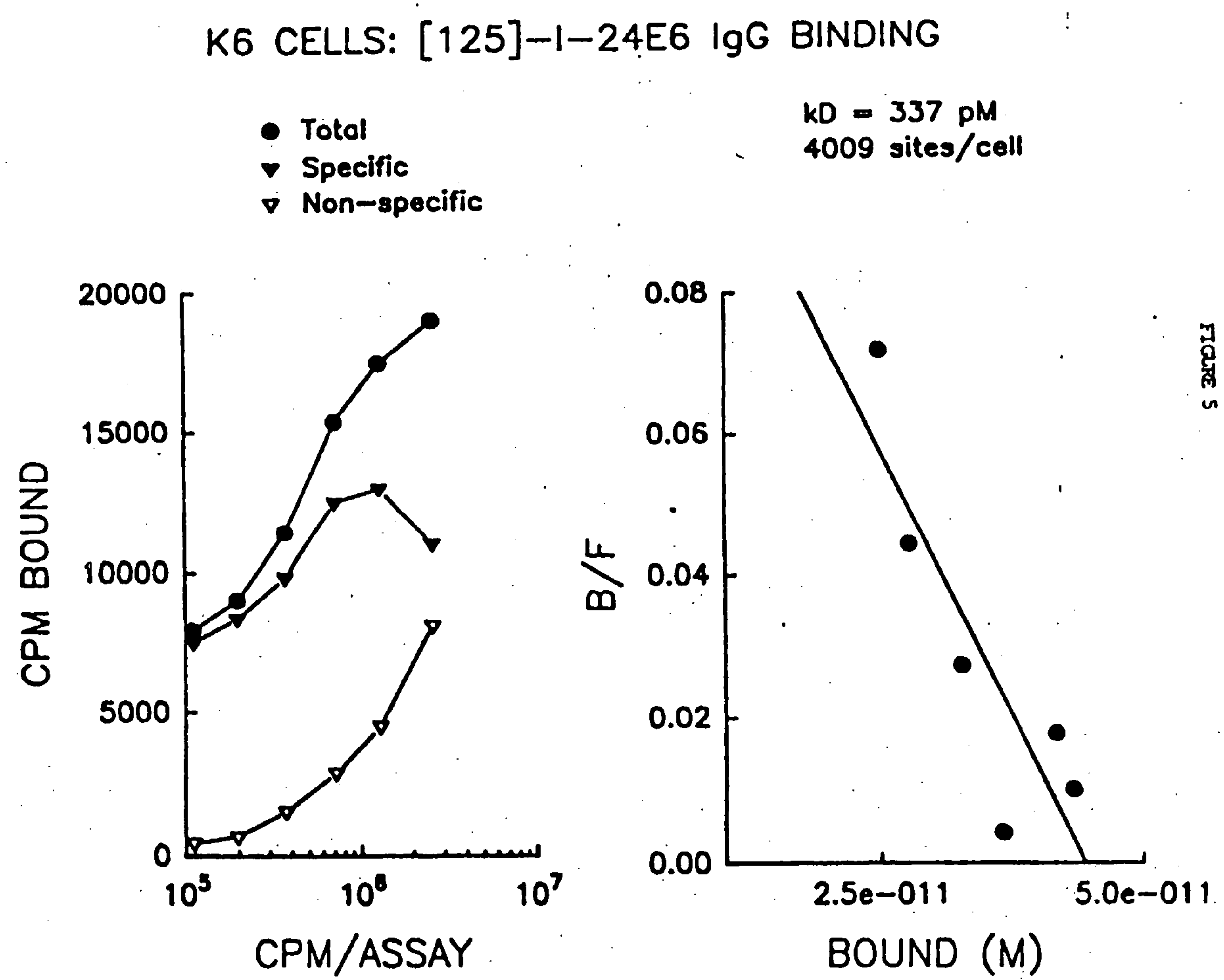

FIGURE 6

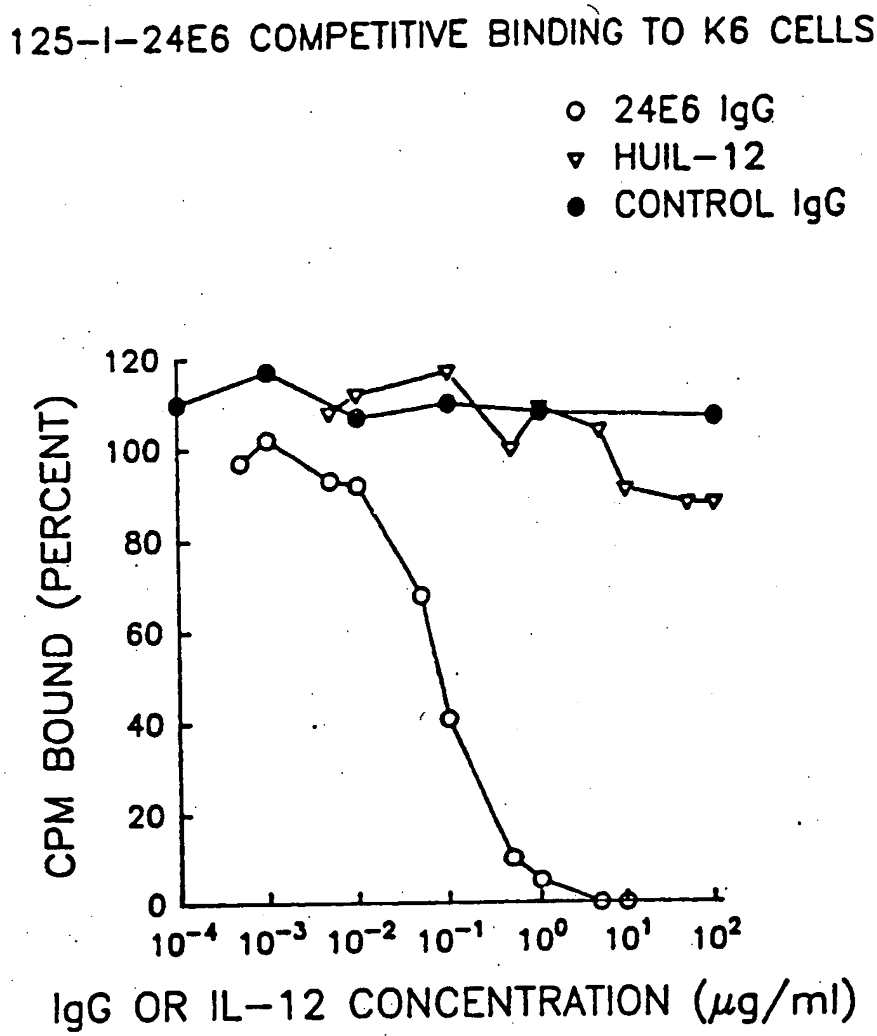
125-I-24E6 COMPETITIVE BINDING TO K6 CELLS
24E6 IgG
HUIL-12
CONTROL IgG
CPM BOUND (PERCENT)
120
100
80
60
40
20
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2}
IgG OR IL-12 CONCENTRATION (μg/ml)

FIGURE 7

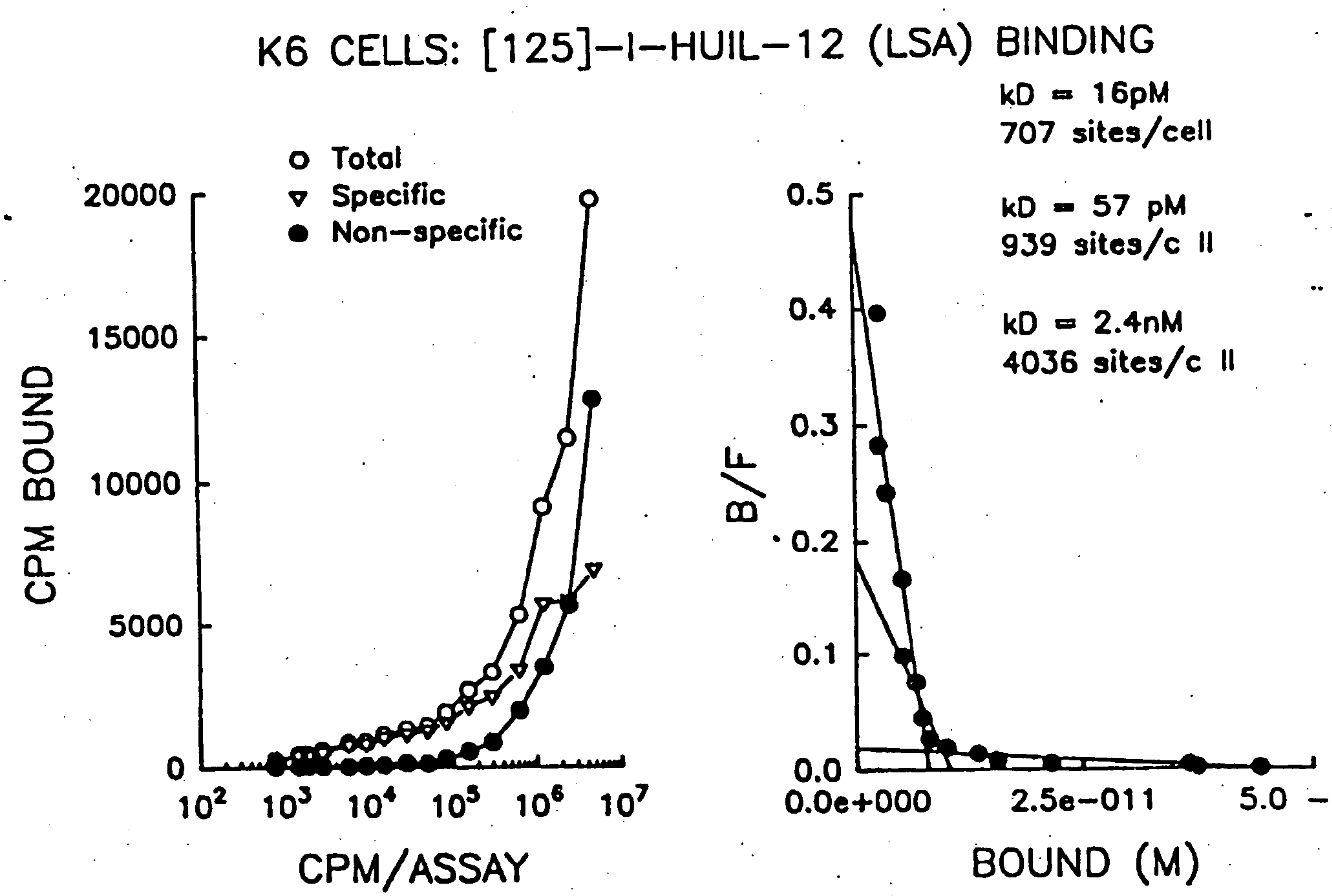

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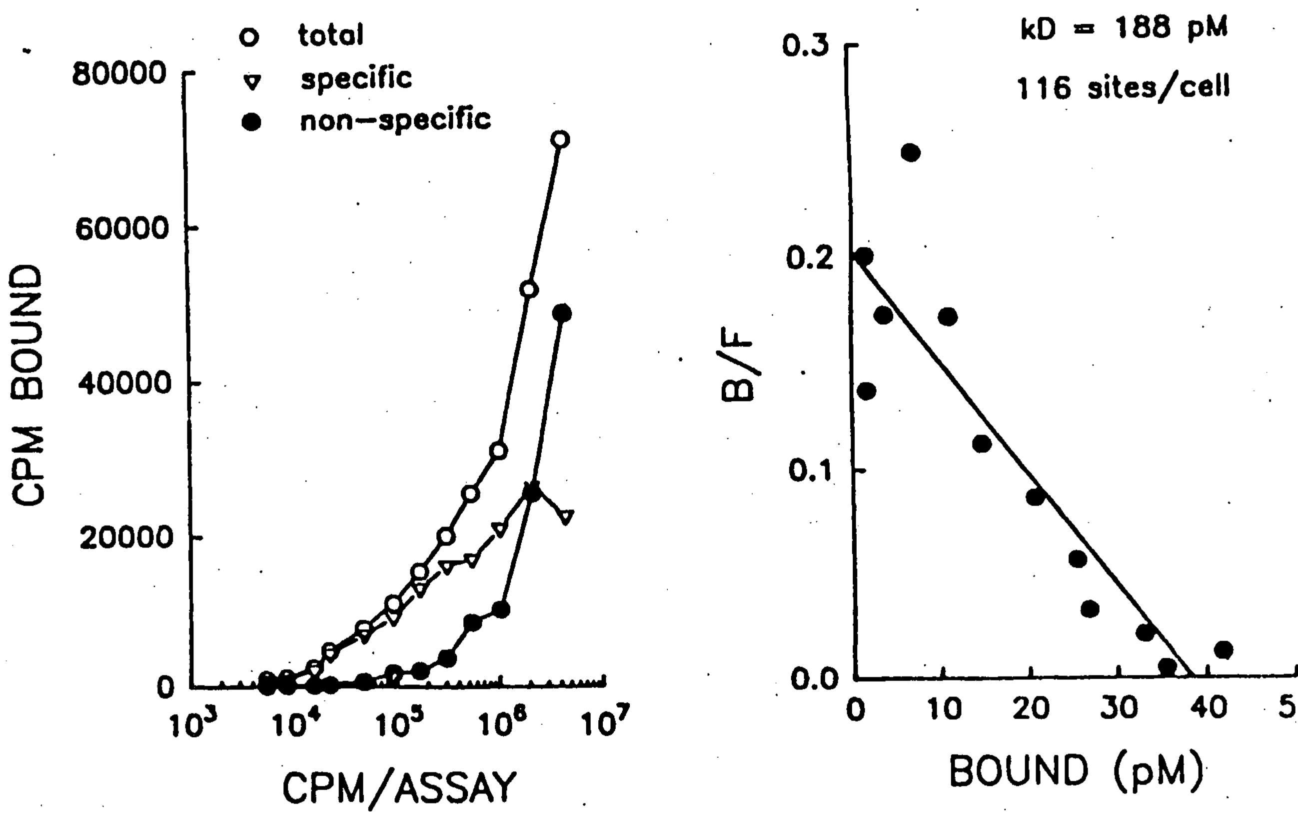
FIGURE 8

[125]-I-HUIL-12 BINDING TO K6 SOLUBLE RECEPTOR



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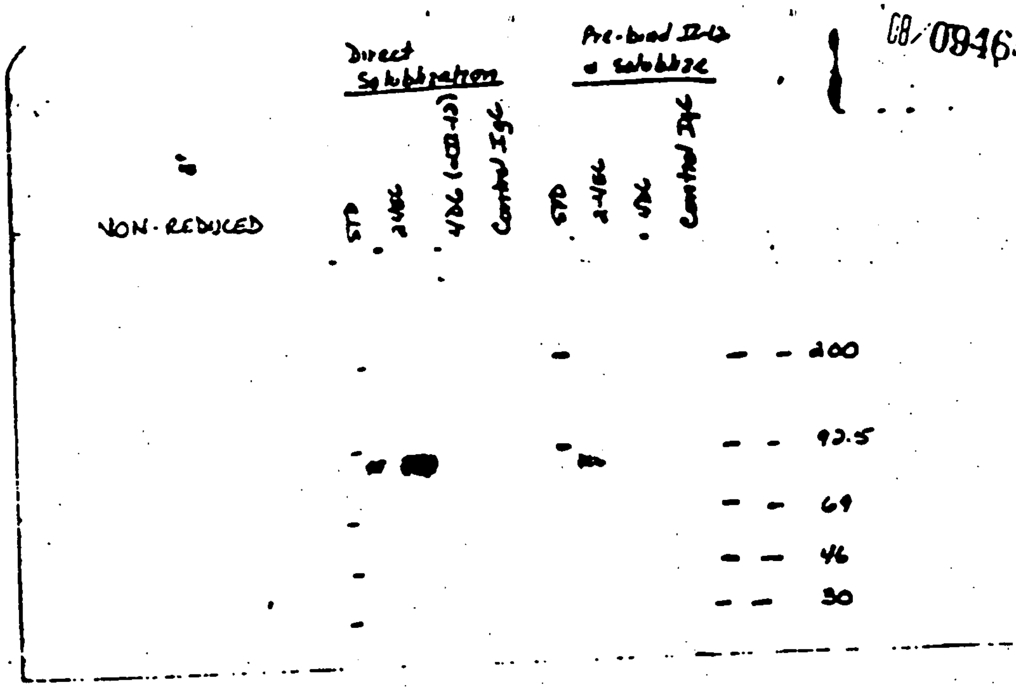

FIGURE 9

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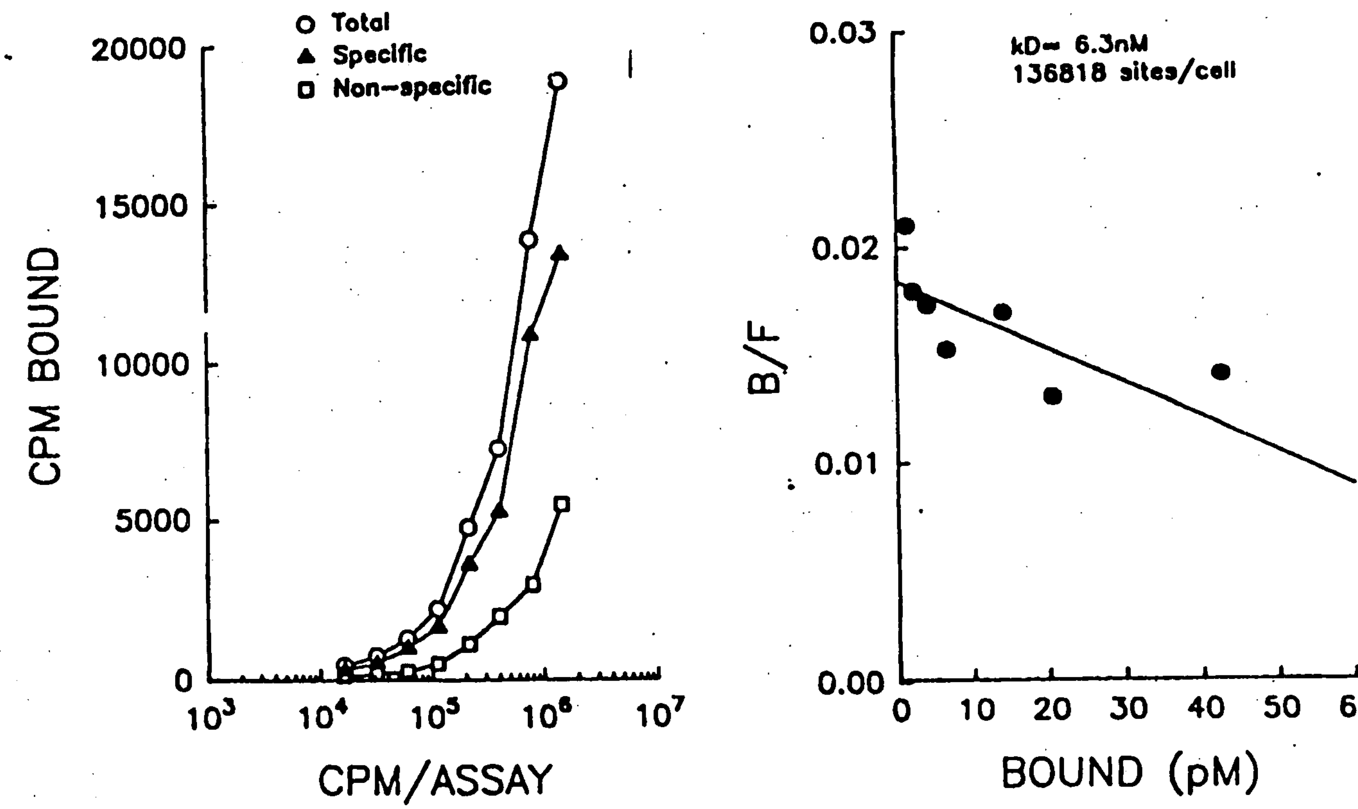
FIGURE 10

COS(H12R) CELLS: [125]-I-HU-IL-12 BINDING



COS(H12R) CELLS: [125]-I-24E6 BINDING

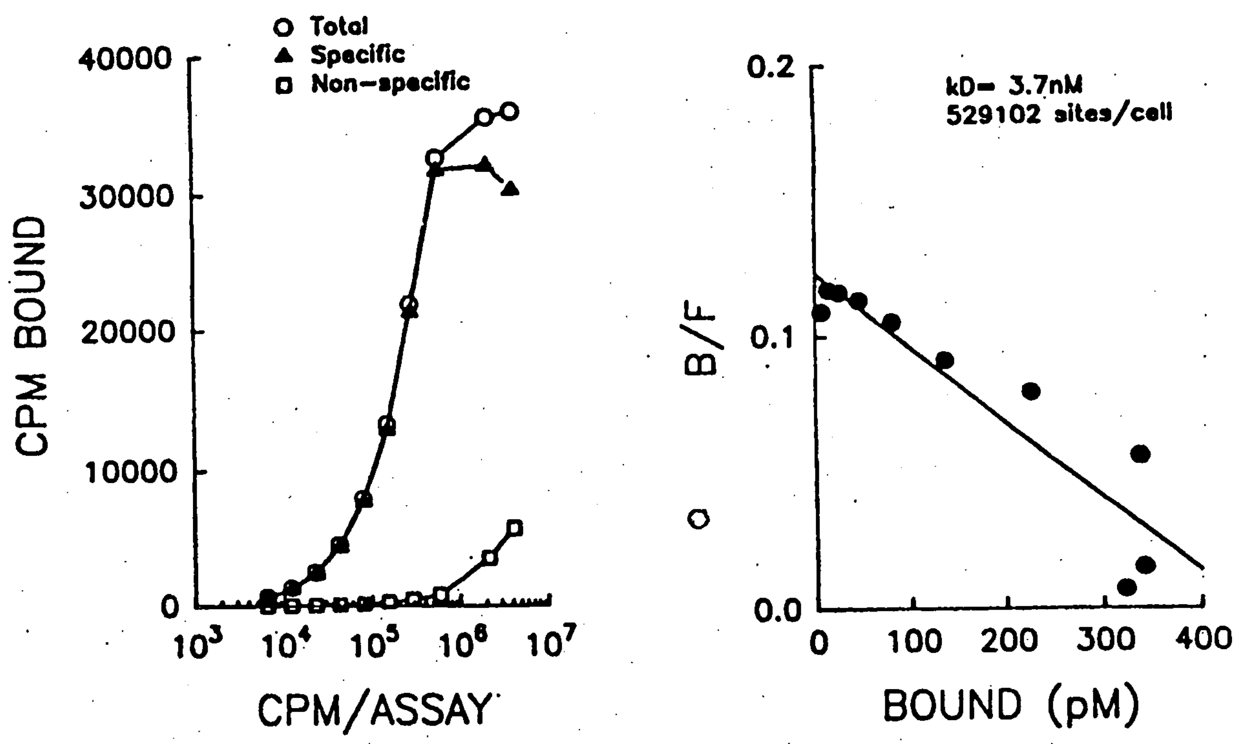


FIGURE 11

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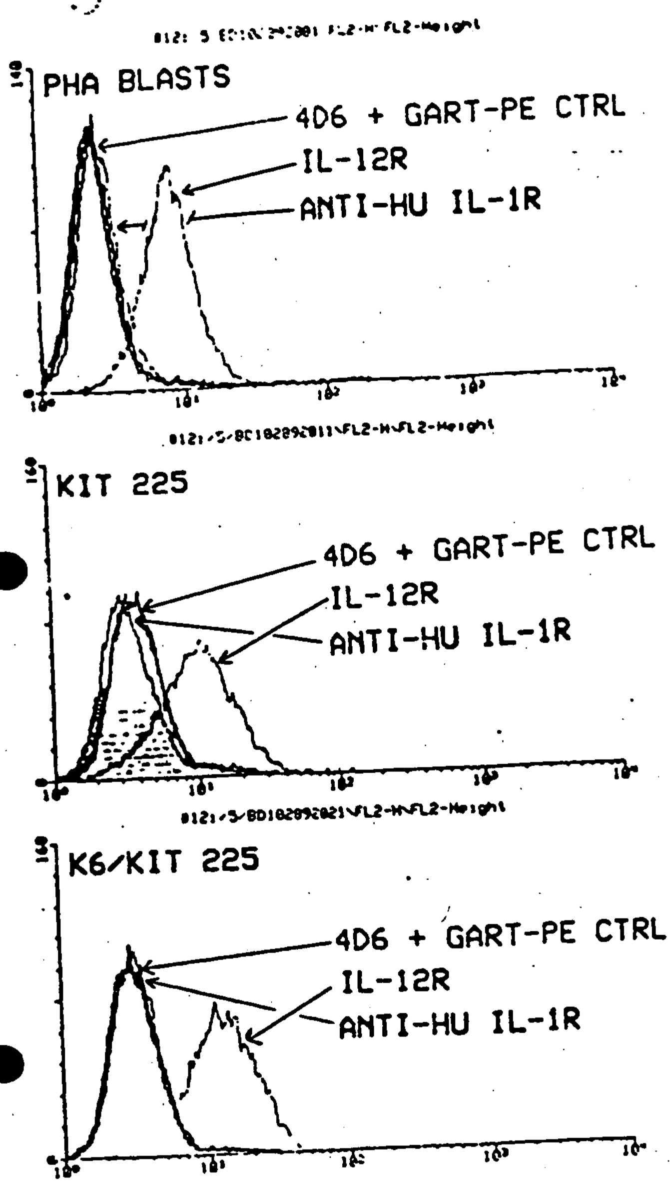
FIGURE 12


PHA BLASTS
4D6 + GART-PE CTRL
IL-12R
ANTI-HU IL-1R
KIT 225
4D6 + GART-PE CTRL
IL-12R
ANTI-HU IL-1R
K6/KIT 225
4D6 + GART-PE CTRL
IL-12R
ANTI-HU IL-1R